RESEARCH COMMUNICATION

Evidence that stimulation of plasma-membrane Ca^{2+} inflow is an early action of glucagon and dibutyryl cyclic AMP in rat hepatocytes

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The ability of glucagon (1 nM) and of dibutyryl cyclic AMP (50 μM) to increase cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in Fura-loaded rat hepatocytes was examined in a system wherein Ca^{2+} inflow was induced by the re-admission of excess Ca^{2+} to a nominally Ca^{2+} -free medium. An increase in $[\text{Ca}^{2+}]_i$ did not occur in the absence of either agonist, but did so after co-addition of either agonist with Ca^{2+} . Increasing the time between addition of dibutyryl cyclic AMP (or of glucagon) and Ca^{2+} led to increases in $[\text{Ca}^{2+}]_i$; half-maximal and maximal increases were observed at 0 s (i.e. at co-addition) and 5–7 s respectively. Dibutyryl cyclic

AMP and Ca^{2+} each exhibited a concentration-dependence when their respective concentrations were changed for a fixed time interval between additions. Half-maximal and maximal effects were obtained with 30 μM and 50 μM dibutyryl cyclic AMP and with 0.5 mM and approx. 1 mM Ca^{2+} respectively. The data demonstrate an early action of glucagon and dibutyryl cyclic AMP on $[\text{Ca}^{2+}]_i$. It is argued that the agonist-induced rise in $[\text{Ca}^{2+}]_i$ results from an increase in plasma-membrane Ca^{2+} inflow, an effect that appears to occur much earlier than that on mobilization of internal stores of Ca^{2+} .

INTRODUCTION

Glucagon, or the second messenger cyclic AMP (generated by the action of glucagon on adenylate cyclase), induces numerous metabolic effects in liver. Among these is their well-recognized ability to potentiate the action of Ca^{2+} -mobilizing agonists [see, e.g., Kraus-Friedmann (1986) and references in the reviews by Mauger and Claret (1988) and Altin and Bygrave (1988)]. It is well known that the actions of glucagon (or of cyclic AMP) alone in such mobilization are slower in onset than are those of vasopressin or phenylephrine in hepatocytes (Charest et al., 1983; Combettes et al., 1986; Poggioli et al., 1986; Banhegyi et al., 1991) or in the perfused rat liver (Reinhart et al., 1982; Morgan et al., 1983, 1984; Altin and Bygrave, 1985). On the other hand, co-administration of glucagon (or of dibutyryl cyclic AMP) with vasopressin, phenylephrine or angiotensin in hepatocytes (Morgan et al., 1983, 1984; Mauger et al., 1985; Combettes et al., 1986; Poggioli et al., 1986; Banhegyi et al., 1991) or perfused rat liver (Morgan et al., 1983, 1984; Altin and Bygrave, 1986; Benedetti et al., 1989; Hamada et al., 1992a,b) leads to rates of Ca^{2+} inflow that are much greater than those observed with either glucagon or Ca^{2+} -mobilizing agonists alone.

The mechanism by which this potentiation occurs is unclear, and an aspect of it is addressed in this paper. We report that administration of glucagon or of the permeant analogue of cyclic AMP, dibutyryl cyclic AMP, to rat hepatocytes, induces a response whereby $[\text{Ca}^{2+}]_i$ is stimulated immediately after agonist addition. Evidence is presented to suggest that Ca^{2+} inflow largely contributes to the agonist-induced increase in $[\text{Ca}^{2+}]_i$; this action appears to be distinct from any action of glucagon on the mobilization of internal pools of Ca^{2+} .

EXPERIMENTAL

Hepatocyte isolation and incubation

Hepatocytes were isolated from male Sprague–Dawley rats

(200–250 g, fed *ad libitum*) by collagenase perfusion of the liver (Moldeus et al., 1978). After isolation, the cells were suspended at a density of $3 \times 10^6/\text{ml}$ in Krebs–Henseleit buffer supplemented with 20 mM Hepes, pH 7.4, and containing 10 mM glucose and 2% (w/v) BSA and incubated at 37 °C in rotating round-bottom flasks under an atmosphere of O_2/CO_2 (19:1). Cell viability was always greater than 90%, as judged by Trypan Blue exclusion frequency.

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was assayed with the fluorescent Ca^{2+} indicator Fura 2. Isolated rat hepatocytes ($3 \times 10^6/\text{ml}$) were loaded with 5 μM Fura 2/AM in the presence of 0.05% Pluronic F-127 in a modified Krebs–Henseleit medium containing 1 mM CaCl_2 , 10 mM glucose, 2% BSA and 20 mM Hepes, pH 7.4, for 20 min at 37 °C. Cells were then washed, resuspended in the same medium ($1.5 \times 10^6/\text{ml}$) and further incubated at 25 °C for 20 min to allow complete de-esterification of Fura 2/AM. Immediately before the measurement of $[\text{Ca}^{2+}]_i$, cells were quickly washed and resuspended ($0.75 \times 10^6/\text{ml}$) in the same medium as above but with 0.2% BSA.

Reaction mixtures contained, in a final volume of 2.0 ml: Krebs–Henseleit buffer, 0.3% BSA, 10 mM glucose and approx. 1.6×10^6 cells; the pH was 7.4. The experiments were carried out according to the Ca^{2+} -free/ Ca^{2+} -reintroduction protocol. EGTA (usually 2 mM) was first added, and subsequently Ca^{2+} (usually 3 mM) was added. Changes in $[\text{Ca}^{2+}]_i$ were monitored by measuring the fluorescence emission at 509 nm with a fluorimeter (Perkin–Elmer model LS-3B) with the excitation wavelength positioned at 340 nm and equipped with a recorder, a temperature-controlled cuvette holder (20 °C) and a magnetic stirrer. In most experiments fluorescence was measured with one excitation wavelength only. However, control experiments were also conducted by point measurement of the ratio of Fura 2 fluorescence (506 nm emission) with excitation at 340 and 380 nm (Fulceri et

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; Fura 2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)ethane-*NNN'*-tetra-acetic acid; Fura 2-AM, Fura 2-acetoxymethyl ester.

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Figure 1 Increase in $[Ca^{2+}]_i$ by dibutyryl cyclic AMP after addition of Ca^{2+} to hepatocytes suspended in a Ca^{2+} -free medium

Changes in $[Ca^{2+}]_i$ of Fura 2-loaded hepatocytes (0.75×10^6 /ml) were monitored as reported in the Experimental section. Initially Ca^{2+} was 1 mM. This was decreased to nominally zero by addition of EGTA (E; 2 mM) at the times indicated. Ca^{2+} (C; 3 mM) was added 2 min later, after, at the times indicated and other than in (a), the addition of 50 μ M dibutyryl cyclic AMP (dbcAMP) (b, dibutyryl cyclic AMP was added 60 s after Ca^{2+} ; c, co-administration of dibutyryl cyclic AMP and Ca^{2+} ; d, dibutyryl cyclic AMP added 5 s before Ca^{2+}). The rapid deflections in fluorescence after EGTA and Ca^{2+} additions were due to Fura 2 (free acid) that had leaked from cells. The $[Ca^{2+}]_i$ scale was calculated by calibration measurements made at the end of the experiment and therefore is representative of the values shown after Ca^{2+} re-introduction. The traces are representative of numerous experiments undertaken.

al., 1991); the values obtained by point measurement were fully consistent with those obtained by continuously monitoring fluorescence with excitation fixed at 340 nm as described above. In parallel samples to which no fura 2/AM was added, it was verified that all the treatments employed caused only minor variations in cell autofluorescence. Moreover, any possible fluorescence changes that might be potentially attributable to, e.g., changes in cell volume of the hepatocytes, are rendered unlikely by the report of Gaussin et al. (1992), showing that cell shrinkage follows, by some minutes, the short-term effects of glucagon in hepatocytes. At the end of any experiment, calibration values for maximum and minimum fluorescence were obtained by permeabilization of hepatocytes with digitonin (10 μ g/ml), followed by addition of 5 mM EGTA and Tris to adjust the pH of the medium to 8. An equilibrium constant of 135 mM (at 20 °C; see Grynkiewicz et al., 1985) for the Ca^{2+} -Fura 2 complex was used to calculate $[Ca^{2+}]_i$. Additions to the cuvette were made manually with micro-syringes. Mixing times were estimated to be less than 1 s.

Materials

Dibutyryl cyclic AMP was from Sigma. Fura-2/AM and Pluronic F-127 were from Molecular Probes. Collagenase (EC 3.4.24.3) and digitonin were from Boehringer. All other chemicals were of analytical grade.

RESULTS

The experimental system adopted to measure Ca^{2+} inflow involved decreasing the external $[Ca^{2+}]$ in the reaction medium to nominally zero by the addition of excess EGTA and then later to

add an excess of Ca^{2+} to re-establish a substantial Ca^{2+} gradient across the plasma membrane. Figure 1 shows the nature of the response in $[Ca^{2+}]_i$. After addition of Ca^{2+} at 2 min after EGTA (Figure 1a), there is little change in $[Ca^{2+}]_i$, indicating a virtual absence of time-dependent Ca^{2+} inflow. Addition of dibutyryl cyclic AMP after re-admission of Ca^{2+} (Figure 1b) induces a slow increase in $[Ca^{2+}]_i$, after a lag period of some 30 s; this returns to basal values by approx. 120 s. Figure 1(c) shows that when dibutyryl cyclic AMP is added simultaneously with Ca^{2+} , there occurs a progressive increase in $[Ca^{2+}]_i$ that continues for approx. 60 s. Figure 1(d) shows that when dibutyryl cyclic AMP is added 5 s before Ca^{2+} , the rate of Ca^{2+} inflow is further enhanced. A similar enhancement of $[Ca^{2+}]_i$ induced by glucagon or dibutyryl cyclic AMP occurred when the intracellular Ca^{2+} pool was first depleted with 1 μ M thapsigargin (results not shown).

Data in Figure 2(a) have been collated from a number of different traces in which the interval between additions of dibutyryl cyclic AMP (or glucagon) and Ca^{2+} was varied for the times shown. The data were gathered from numerous individual runs and in which the rate of change in $[Ca^{2+}]_i$ was measured, as illustrated by the discontinuous lines shown in Figure 1. The effects of both dibutyryl cyclic AMP and glucagon are extremely rapid; as intimated above, even the simultaneous addition of either agent with Ca^{2+} is already sufficient to activate the response by near half-maximal rates; maximal rates are observed by 5–7 s.

Figure 2(b) shows how $[Ca^{2+}]_i$ changes as the concentration of dibutyryl cyclic AMP was varied for a fixed (10 s) time interval between its addition and that of Ca^{2+} . Half-maximal and near-maximal responses are attained at approx. 30 μ M and 50 μ M dibutyryl cyclic AMP respectively.

If the responses observed above are driven by the Ca^{2+} gradient across the plasma membrane, we should observe a response that becomes attenuated as this gradient is decreased, for a given time

Figure 2 Some properties of the increase in $[Ca^{2+}]_i$ induced by dibutyryl cyclic AMP

The experimental system was the same as that described in Figure 1. To obtain the data shown here, rates of change of $[Ca^{2+}]_i$ were measured from traces obtained like those shown in Figure 1 but with the variations indicated below. Rates of change were measured in arbitrary units by drawing lines of best fit similar to the discontinuous lines shown in Figure 1. In (a) the time (in s) between addition of 50 μM dibutyryl cyclic AMP or 1 nM glucagon and Ca^{2+} was varied as shown; in (b) varying dibutyryl cyclic AMP additions were made 10 s before Ca^{2+} re-admission, and in (c) Ca^{2+} added was varied to exceed $[EGTA]$ as shown, with 50 μM dibutyryl cyclic AMP added 10 s before this Ca^{2+} .

between addition of dibutyryl cyclic AMP and Ca^{2+} . Data in Figure 2(c) show this is the case. The concentration of re-admitted Ca^{2+} was varied as indicated, and the time between additions of dibutyryl cyclic AMP and Ca^{2+} was fixed at 10 s. Little change in $[Ca^{2+}]_i$ is observed until approx. 0.25 mM Ca^{2+} is added; half-maximal and maximal changes are seen at approx. 0.5 mM and 1–1.5 mM added Ca^{2+} respectively. The $[Ca^{2+}]_i$ -dependence observed here is similar to the dependence of plasma-membrane Ca^{2+} cycling on external Ca^{2+} observed in the perfused rat liver (Reinhart et al., 1984) and to the dependence of the

initial rate of $^{45}Ca^{2+}$ uptake on external $[Ca^{2+}]$ in hepatocytes (Mauger et al., 1985).

DISCUSSION

The technique used in this work to determine changes in $[Ca^{2+}]_i$ after the rapid re-admission of Ca^{2+} to a medium containing nominally zero free Ca^{2+} has been used previously as a means of evaluating plasma-membrane Ca^{2+} inflow (see, e.g., Putney, 1990). The technique is relatively simple and allows detection of any Ca^{2+} inflow that is rapid in onset. One serious limitation in any work of this nature, however, is the inability to exclude without doubt any contribution that intracellular Ca^{2+} stores might make to a change in $[Ca^{2+}]_i$. The evidence presented here supporting the contention that Ca^{2+} inflow is a major contributor to the observed increases in $[Ca^{2+}]_i$ therefore cannot be seen to be conclusive.

The Ca^{2+} -free/ Ca^{2+} -readmission technique showed that addition either of dibutyryl cyclic AMP or of glucagon to rat hepatocytes rapidly induces an increase in $[Ca^{2+}]_i$. With both agents, half-maximal responses are induced instantaneously and maximal responses by approx. 7 s. The driving force for this inflow appears to be the plasma-membrane $[Ca^{2+}]$ gradient, since the magnitude of this gradient determined the rate of agonist-induced Ca^{2+} inflow. Moreover, these increases in Ca^{2+} fluxes increased as [dibutyryl cyclic AMP] was increased (half-maximal effects were seen at approx. 30 μM dibutyryl cyclic AMP) and occurred independently of the action of exogenously added Ca^{2+} -mobilizing agonists that generate $Ins(1,4,5)P_3$. The effect of glucagon was not determined in detail here, but 1 nM glucagon induced a rate increase that was only slightly less than that induced by 10 nM glucagon (results not shown). Of especial note too was the observation that addition of dibutyryl cyclic AMP to a medium containing Ca^{2+} exhibited a pattern of $[Ca^{2+}]_i$ that was different from the rapid responses observed (Figure 1b). This slower response to dibutyryl cyclic AMP most likely represents mobilization of intracellular Ca^{2+} pools (see, e.g., Kass et al., 1990, and below).

Although we interpret this increase in $[Ca^{2+}]_i$ to reflect an increase in inflow of Ca^{2+} from the external medium, it could be argued that the responses observed here reflect an effect(s) of dibutyryl cyclic AMP that lead to the depletion of internal Ca^{2+} stores that would create an intracellular Ca^{2+} 'vacuum', which then in turn would be a major determinant of plasma-membrane Ca^{2+} inflow (e.g. Putney, 1990; and see Irvine, 1992). Indeed, evidence exists for mobilization of Ca^{2+} from intracellular store(s) by dibutyryl cyclic AMP (Altin and Bygrave, 1986; Combettes et al., 1986; Mine et al., 1988; Figure 1b). Several lines of evidence make this an unlikely possibility in this instance. First, the onset of the increase in $[Ca^{2+}]_i$ reflecting such depletion appears relatively later, at approx. 10–15 s, with maximal changes seen only 50–60 s after dibutyryl cyclic AMP (Combettes et al., 1986; Banhegyi et al., 1991) or glucagon addition (Charest et al., 1983). Second, in contrast with the data in Figure 2(c), we have observed (F. L. Bygrave, A. Gamberucci, R. Fulceri and A. Benedetti, unpublished work) that the extent of dibutyryl cyclic AMP-induced mobilization of intracellular Ca^{2+} increases as the external $[Ca^{2+}]$ is lowered. Third, and consistent with indications that more than one Ca^{2+} -inflow pathway appears to exist in the liver plasma membrane (e.g. Altin and Bygrave, 1987; Llopis et al., 1992), depletion by thapsigargin of intracellular Ca^{2+} pool(s), but not by glucagon addition, activates Mn^{2+} entry (Kass et al., 1990). However, since in their experiments prior administration of glucagon (or dibutyryl cyclic AMP) led to enhanced vasopressin- or thapsigargin-stimulated entry of Mn^{2+} , it would

appear that dibutyl cyclic AMP might have multiple actions on plasma-membrane Ca^{2+} fluxes. Fourth, in other experiments (F. L. Bygrave, A. Gamberucci, R. Fulceri and A. Benedetti, unpublished work), we have shown that, after depletion of the internal Ca^{2+} pools by thapsigargin, re-admission of Ca^{2+} with dibutyl cyclic AMP or glucagon as shown here also leads to similar stimulatory effects on Ca^{2+} inflow. Fifth, Mauger et al. (1985) showed that the initial rate of $^{45}\text{Ca}^{2+}$ uptake by hepatocytes is stimulated by glucagon. Sixth, Staddon and Hansford (1986), using a technique similar to that used here, showed that glucagon stimulated net inflow of Ca^{2+} into hepatocytes; in these latter experiments, the time interval between glucagon addition and Ca^{2+} re-admission was considerably greater than that used in the present work.

The fact that both glucagon and dibutyl cyclic AMP induced a similar pattern of action renders it unlikely also that the effects of glucagon are attributable to any InsP_3 that might be generated from glucagon in the short time intervals adopted here (see Wakelam et al., 1986).

Apart from the rapidity of onset, our experiments reveal little about the mechanistic nature of the stimulatory effects induced by dibutyl cyclic AMP. Although various authors (see references above) have suggested that dibutyl cyclic AMP might act on the hepatocyte plasma membrane in a manner similar to its action on Ca^{2+} channels of excitable cells (Hosey and Lazdunski, 1988), evidence for this to date is scant. On the other hand, it is known that dibutyl cyclic AMP is able to phosphorylate some proteins in the plasma membrane (Vargas et al., 1982). Whether any of these proteins are components of or are associated with the hepatocyte plasma-membrane Ca^{2+} -inflow pathways has yet to be determined.

Finally, this early action of dibutyl cyclic AMP on plasma-membrane Ca^{2+} inflow might be seen as a means of priming the inflow 'transporter(s)' in preparation for the triggering action of InsP_3 or a metabolite of it generated by the action of Ca^{2+} -mobilizing agonists (see, e.g., Irvine, 1992). In the perfused rat liver, for example, simultaneous administration of dibutyl cyclic AMP and such agonists lead to an instantaneous inflow of Ca^{2+} that is much greater than that induced by the Ca^{2+} -mobilizing agonists alone (Altin and Bygrave, 1986). It is possible that endogenous levels of cyclic AMP are acting continuously in this way and that this could be important in the regulation of Ca^{2+} -dependent physiological events such as bile secretion, for

instance, which recent work has revealed is sensitive to the synergistic action of glucagon and Ca^{2+} -mobilizing agonists (Hamada et al., 1992a,b).

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